Remarks

Reconsideration and withdrawal of the rejections of the claims, in view of the amendments and remarks herein, is respectfully requested. Claims 1, 45, 47, and 63 are amended, claims 10, 13, 16-17, 19, 22-23, 40, 46, 48-59, and 65-66 are canceled, and claims 67-68 are added. The amendments are intended to advance the application and are not intended to concede to the correctness of the Examiner's position or to prejudice the prosecution of the claims prior to amendment, which claims are present in a continuation of the above-identified application. Claims 1-9, 11-12, 14-15, 18, 20-21, 24-39, 41-45, 47, 60-64, and 67-68 are pending.

Claims 10, 13, 16-17, 19, 22-23, 40, 46, 48-59, and 65-66 are canceled solely in response to the Restriction Requirement, and without prejudice to their prosecution in an appropriately-filed divisional application.

Amended claim 1 is supported by originally-filed claim 1, and at page 35, lines 5-16, page 35, line 24-page 36, line 5, and page 60, lines 23-26 of the specification.

Amended claim 45 is supported by originally-filed claim 45.

Amended claim 47 is supported by originally-filed claim 47, and at page 20, lines 21-27, page 35, lines 5-16, page 35, line 24-page 36, line 5, and page 60, lines 23-26 of the specification.

Amended claim 63 is supported by originally-filed claim 63.

New claim 67 is supported by originally-filed claims 1, 9 and 14.

New claim 68 is supported by originally-filed claim 1.

The 35 U.S.C. § 112, Second Paragraph, Rejections

The Examiner rejected claims 1-9, 11-12, 14-15, 18, 20-21, 24-39, 41-45, 47, 54, and 63 under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicant regards as the invention. This rejection, as it may be maintained with respect to the pending claims, is respectfully traversed.

In particular, the Examiner asserts that i) the phrase "3-fold fewer transcription regulatory sequences relative to the average number of such sequences resulting from

random selections of codons at the codons which differ" in claim 1 is vague and indefinite, as the specification provides no method for determining the average number of such sequences resulting from random selections of codons at the codons which differ; ii) the phrase "3-fold fewer transcriptional regulatory sequences relative to a vector comprising a parent nucleic acid sequence" in claim 63 is confusing, as such a calculation is impossible without knowing all the possible sequences which are considered to be "transcriptional regulatory sequences"; iii) the phrase "A reporter gene expression kit" in claim 45 is confusing as the expression vector recited does not necessarily encode a reporter gene; and iv) the phrase "stringent conditions" in claim 47 is indefinite as the specification does not define what conditions are "stringent."

The amendments to claims 45 and 47 render the § 112(2) rejection as it relates to those claims moot.

With respect to claim 63, the Examiner is requested to note that claim 63 specifies the transcriptional regulatory sequences, i.e., transcription factor binding sequences, intron splice sites, poly(A) addition sites and promoter sequences.

With respect to the phrase "3-fold fewer transcription regulatory sequences relative to the average number of such sequences resulting from random selections of codons at the codons which differ" in claim 1, it is Applicant's position that the metes and bounds of that phrase in the context of the claims would be clear to one of ordinary skill in the art. First, there are 64 codons, 61 of which encode an amino acid. Claim 1 recites that the polypeptide encoded by the synthetic nucleic acid molecule has at least 85% sequence identity to the polypeptide encoded by the wild type nucleic acid sequence. Thus, the vast majority of the codons which differ encode the same amino acid.

Moreover, for any given set of possible sequence motifs, by random chance alone, a particular sequence would likely contain transcriptional regulatory sequences, and the average occurrence of those sequences within a coding region could be readily determined either manually or via computer. Indeed, a coding region analysis would show a Poisson type distribution curve of the transcriptional regulatory motifs. Given this information, it would be apparent to one skilled in the art that the probability of

finding 3-fold fewer motifs in a coding region containing, for example, containing 40 or more transcriptional regulatory motifs, is around 1:1,000,000. Therefore, if 3-fold fewer motifs were found in a given coding sequence, it would be apparent that that occurrence was not random.

Therefore, it is Applicant's position that the phrase "3-fold fewer transcription regulatory sequences relative to the average number of such sequences resulting from random selections of codons at the codons which differ" in claim 1 is clear. However, to advance the application, claim 1 no longer recites the phrase at issue.

Accordingly, withdrawal of the § 112(2) rejections is respectfully requested.

The 35 U.S.C. § 112, First Paragraph, Rejections

The Examiner rejected claims 1-6, 14-15, 20-21, 24-33, 35-39, 41-45, 47, 54, 60-61, and 63 under 35 U.S.C. § 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one of skill in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention (a "written description" rejection). The Examiner also rejected claims 1-6, 14-15, 20-21, 24-33, 35-39, 41-45, 47, 54, 60-61, and 63 under 35 U.S.C. § 112, first paragraph (an enablement rejection). These rejections, as they may be maintained with respect to the pending claims, are respectfully traversed.

Claim 1 is directed to a synthetic nucleic acid molecule comprising at least 300 nucleotides of a coding region for a polypeptide, wherein the codon composition and transcription regulatory sequence composition of the synthetic nucleic acid molecule is different than that of a wild type nucleic acid sequence encoding a polypeptide which has at least 85% sequence identity to the polypeptide encoded by the synthetic nucleic acid molecule, wherein the codons which differ are selected so as to result in the synthetic nucleic acid molecule having a codon composition differing at more than 25% of the codons from the wild type nucleic acid sequence and having a reduced number of transcription factor binding sequences and optionally a reduced number of sequences selected from the group consisting of intron splice sites, poly(A) addition sites and promoter sequences, and wherein the synthetic nucleic acid molecule has reduced

aberrant transcription relative to the transcription of the wild type nucleic acid sequence. Claims 35 and 36 are directed to a plasmid or expression vector, respectively, comprising the synthetic nucleic acid molecule of claim 1, and claims 44 and 45 are directed to a host cell or kit, respectively, comprising the expression cassette of claim 36. Claim 47 is directed to a polynucleotide which hybridizes under medium stringency hybridization conditions to SEQ ID NO:22 (Rluc-final), SEQ ID NO:9 (GRver5.1), SEQ ID NO:18 (RD156-1H9), SEQ ID NO:297 (GRver5.1), SEQ ID NO:301 (RD156-1H9), or the complement thereof. Claim 63 is directed to a vector comprising a gene of interest and backbone sequences, wherein the backbone sequences comprise a synthetic nucleic acid molecule having 3-fold fewer sequences selected from the group consisting of transcription factor binding sequences, intron splice sites, poly(A) addition sites and promoter sequences relative to a parent nucleic acid sequence.

With respect to the written description rejection, the Examiner asserts that the specification does not contain any disclosure of the function of all nucleic acids within the scope of the claimed genera, which encompasses many functionally unrelated DNAs, and that the specification discloses only a few species of the claimed genera which is insufficient to put one of skill in the art in possession of the attributes and features of all species within the claimed genus.

To provide an adequate written description for a claimed genus, the specification can provide a sufficient description of a representative number of species by an actual reduction to practice, reduction to drawings or by a disclosure of relevant, identifying characteristics, i.e., by a structure or other physical and/or chemical properties, by functional characteristics coupled with a known or disclosed correlation between function and structure, or by a combination of such identifying characteristics. Guidelines for Examination of Patent Applications under the 35 U.S.C. § 112(1) Written Description Requirement, Fed. Reg., 66, 1099 (2001). Satisfactory disclosure of a representative number of species depends on whether one skilled in the art would recognize that Applicant was in possession of the necessary common attributes or features of the elements possessed by members of the genus. Guidelines for Examination of Patent Applications under the 35 U.S.C. § 112(1) Written Description

Requirement, Fed. Reg., 66, 1099 (2001).

The specification provides a detailed description of the preparation of synthetic nucleic acid molecules having codon modifications, i.e., modifications which result in RNA having improved translatability in a particular host cell, and decreased transcriptional regulatory sequences, i.e., modifications which result in enhanced transcription of a synthetic DNA molecule, and so reflect changes which occur during natural selection (page 33, line 6-page 35, line 4 and page 38, lines 5-30). Codon usage and transcriptional regulatory sequences in various organisms are known (page 11, lines 28-30 and page 38, lines 20-23).

For instance, the specification discloses that a *Renilla* luciferase gene and a yellow-green click beetle luciferase gene (YG#81-6G01) were modified by replacing codons and reducing the number of transcriptional regulatory sequences (Examples 1-3). Codons were generally substituted with mammalian high-usage codons and not with mammalian low-usage or *E. coli* low-usage codons, so that in most cases the substituted codons did not add transcription regulatory sequences (page 53, lines 8-10; page 66, lines 24-26). Two synthetic *Renilla* luciferase sequences (Rluc ver 2 and Rluc final) and 14 synthetic click beetle luciferase sequences (GRver2, GRver3, GRver4, GRver5, GR6, GRver5.1, RDver2, RDver3, RDver4, RDver5, RD7, RDver5.1, RDver5.2 and RD156-1H9) are disclosed. The synthetic genes tested had enhanced expression and reduced levels of spurious transcription relative to the parental genes when introduced into mammalian host cells (Tables 9 and 12).

Clearly, Applicant has disclosed relevant, identifying characteristics of the claimed synthetic nucleic acid molecules, e.g., functional characteristics coupled with a known or disclosed correlation between function and structure, i.e., synthetic nucleic acid molecules that comprise codon substitutions and reduced transcription factor binding sequences, intron sites, poly(A) addition sites and promoter sequences that result in improved expression, including improved transcription, of the synthetic nucleic acid molecule.

Moreover, given the numerous synthetic sequences disclosed in the specification, one skilled in the art would recognize that Applicant was in possession of the necessary

common attributes or features of the elements possessed by members of the genus. Therefore, Applicant's specification satisfies the written description requirement of 35 U.S.C. § 112(1).

As for enablement, the Examiner asserts that the specification, while being enabling for a variant of a parent DNA molecule encoding a polypeptide identical to a polypeptide encoded by the parent DNA and having more than 25% of the codons altered and having at least 3-fold fewer transcription regulatory sequences than the parent nucleic acid or to any nucleic acid which will hybridize to SEQ ID NO:9 under high stringency conditions and encoding a polypeptide having luciferase activity, does not reasonably provide enablement for any variant DNA molecule encoding a polypeptide having at least 85% identity to a wild type polypeptide, and having more than 25% of the codons altered and at least 3-fold fewer transcription regulatory sequences than the parent nucleic acid, or to any nucleic acid which hybridizes to SEQ ID NO:9. In particular, the Examiner alleges that the specification does not establish regions of the protein structure which may be modified without effecting activity.

The Examiner concedes that recombinant and mutagenesis techniques are known. It is Applicant's position that it is well within the skill of the art worker to prepare a nucleic acid molecule encoding a polypeptide having at least 85% identity to a reference polypeptide, which nucleic acid molecule has an altered codon composition and optionally other altered sequences, e.g., see Iannaconne et al. (Plant Mol. Biol., 34:485 (1997)), Sherf et al. (U.S. Patent No. 5,670,356) and Zolotukhin et al. (U.S. Patent No. 5,874,304), documents cited against the claims under 35 U.S.C. §§ 102 and/or 103.

Moreover, it is Applicant's position that it is well within the skill of the art to selectively or randomly substitute amino acids in a particular protein and screen the resulting mutated proteins for a desired activity (for example, for "random" mutagenesis, see Arnold et al. (Chem. Engeering Science, 51:5091 (1996)), WO 99/14336, Stemmer et al. (Proc. Natl. Acad. Sci. U.S.A., 91:10747 (1994)), and Zhao et al. (Proc. Nat. Acad. Sci., 94:7997 (1997)), and for selected mutagenesis, see Sherf et al. and Zolotukhin et al., documents cited against the claims under 35 U.S.C. § 103, Maranville et al. (Eur. J.

Biochem., 267:1495 (2000)), Kao et al. (FEBS Lett., 466:87 (2000)), Petit et al. (BBRC, 251:714 (1998)), Seol et al. (Biol. Chem., 378:1205 (1997)), McWherter et al. (J. Biol. Chem., 272:1874 (1997)), and Walker et al. (J. Virol., 69:8173 (1995)) (a copy of each is enclosed herewith). In this regard, the Examiner is also requested to note that certain amino acid substitutions were introduced to codons in synthetic click beetle luciferases genes: GRver2-GRver5, and GRver5.1 have 1 amino acid substitution (related to a substitution associated with green light) relative to parent sequence YG#81-6G01; RDver2-RDver5 and RDver5.1 have 4 amino acid substitutions (related to substitutions associated with red light) relative to YG#81-6G01; RDver5.2 has 5 amino acid substitutions (related to substitutions associated with red light and improved spectral properties) relative to YG#81-6G01; and RD156-1H9 has 9 amino acid substitutions (related to substitutions associated with red light, improved spectral properties and improved luminescence intensity) relative to YG#81-6G01.

Further, the fact that the outcome of a synthesis/screening program may be unpredictable is precisely why a screening program is carried out. The Examiner simply cannot reasonably contend that a screening program to locate biomolecules with target biological or physical properties would not be carried out by the art because the results cannot be predicted in advance. In fact, the Federal Circuit has explicitly recognized that the need, and methodologies required, to carry out extensive synthesis and screening programs to locate bioactive molecules do not constitute undue experimentation. In re Wands, 8 U.S.P.Q.2d 1400, 1406-1407 (Fed. Cir. 1988), the Court stated:

> The nature of monoclonal antibody technology is that it involves screening hybridomas to determine which ones secrete antibody with desired characteristics. Practitioners of this art are prepared to screen negative hybridomas in order to find one that makes the desired antibody.

Likewise, practitioners in the art related to the present application would be well-equipped to prepare and screen synthetic nucleic acid molecules, e.g., ones encoding polypeptides with less than 100% identity to a native polypeptide, to determine their properties. See also, Hybritech Inc. v. Monoclonal Antibodies Inc., 231 U.S.P.Q. 81, 84 (Fed. Cir. 1986) (evidence that screening methods used to identify characteristics [of

monoclonal antibodies] were available to art convincing of enablement). Therefore, Applicant's specification is fully enabling.

Thus, withdrawal of the § 112(1) rejections is appropriate and is respectfully requested.

The 35 U.S.C. § 102(b) and § 103(a) Rejections

The Examiner rejected claims 1-3, 6, 34-37, 44, 54, and 60-63 under 35 U.S.C. § 102(b) as being anticipated by Iannacone et al. (Plant Mol. Biol., 34:485 (1997)). The Examiner also rejected claims 1-9, 11-12, 14-15, 20-21, 24-39, 41-45, 54, and 60-63 under 35 U.S.C. § 103(a) as being unpatentable over Sherf et al. (U.S. Patent No. 5,670,356) in view of Zolotukhin et al. (U.S. Patent No. 5,874,304) and Iannacone et al. As these rejections may be maintained with respect to the pending claims, they are respectfully traversed.

Iannacone et al. disclose synthetic *Bacillus thuringienesis* Bt43 genes (abstract). To prepare those genes, Iannacone et al. modified the nucleotide sequence of Bt43 in four target regions to avoid sequences which might destabilize mRNA, sequences such as ATTTA sequences, polyA sequences, splicing sites and A+T strings > 4, and to improve codon usage for plant expression (abstract and page 490). Five constructs, one with the wild type Bt43 gene and four with synthetic Bt43 genes, i.e., BtE, BtF, BtH and BtI (see Figure 2 and Table 1), were introduced to eggplant or *Solanum integrifolium* cultures, and transgenic plants regenerated. Bt43-specific polyA+ RNA in the plants was detected by Northern blot analysis (Figure 4).

It is disclosed in Iannacone et al. that no Bt43-specific bands were detected in lanes for plants with the wild type Bt43 gene even after long exposures (page 491). Interestingly, plants expressing the BtE gene had higher levels of Bt43-specific polyA+ mRNA than plants expressing the BtF gene, a gene which had fewer $(A)_{>4}$ and $(T)_{>4}$ strings, one less ATTTA sequence, and more codons modified relative to the BtE gene. In fact, no full size Bt43-specific mRNA was detected in BtF transgenic plants in contrast to BtE transgenic plants (page 494). And although full length BtE polyA+ RNA was present in BtE transgenic plants, no Bt toxin was produced, leading the authors to

conclude that a 1.2 Kb unmodified domain in the BtE gene is a major candidate for translational blockade (page 494).

The authors of Iannacone et al. conclude that the increased level of Bt43-specific mRNA in BtE and BtF transgenic plants compared to wild type Bt43 transgenic plants could be related to the elimination of destabilizing sequences and that the AUUUA string in wild type Bt43 is a major candidate for the instability and untranslatability of Bt43 mRNA (page 494).

Iannacone et al. do not disclose or suggest a synthetic nucleic acid molecule which has a codon composition and transcription regulatory sequence composition that is different than that of a wild type nucleic acid sequence encoding a polypeptide which has at least 85% sequence identity to the polypeptide encoded by the synthetic nucleic acid molecule, wherein the codons which differ are selected so as to result in a synthetic nucleic acid molecule having a codon composition differing at more than 25% of the codons from the wild type nucleic acid sequence and having a reduced number of transcription factor binding sequences, or a vector with a backbone comprising a synthetic nucleic acid molecule having a reduced number of certain sequences relative to a vector comprising a parent nucleic acid sequence.

Thus, withdrawal of the § 102(b) rejection is respectfully requested.

Sherf et al. disclose a synthetic firefly luciferase gene in which 3 internal palindromic sequences, 5 restriction endonuclease sites, 4 glycosylation sites, and 6 transcription factor binding sites were removed, and codons were altered at sequences specified in Table 2 to codons preferred in mammalian cells, relative to a wild type firefly luciferase gene. It is disclosed that 69 codons out of 549 were modified (column 10, lines 2-4).

Sherf et al. do not teach or suggest a synthetic nucleic acid molecule having a codon composition differing at more than 25% of the codons of a corresponding wild type nucleic acid sequence, or a vector with a backbone comprising a synthetic nucleic acid molecule having a reduced number of particular regulatory sequences relative to a vector comprising a parent nucleic acid sequence.

A humanized version of a green fluorescent protein (GFP) gene is disclosed in

Zolotukhin et al. in which 88/238 of the codons in the gene were altered (column 13, lines 1-4). Zolotukhin et al. do not disclose or suggest a synthetic nucleic acid molecule which has a codon composition and transcription regulatory sequence composition that is different than that of a wild type nucleic acid sequence encoding a polypeptide which has at least 85% sequence identity to the polypeptide encoded by the synthetic nucleic acid molecule, wherein the codons which differ are selected so as to result in a synthetic nucleic acid molecule having a codon composition differing at more than 25% of the codons from the wild type nucleic acid sequence and having a reduced number of transcription factor binding sequences, or a vector with a backbone comprising a synthetic nucleic acid molecule having a reduced number of transcriptional regulatory sequences relative to a vector comprising a parent nucleic acid sequence.

The Examiner asserts that it would have been obvious to further modify the luciferase gene of Sherf et al. to both increase the codon preference for humans and to remove potential polyadenylation sites and splice sites in order to further increase its usefulness as a reporter. The Examiner also asserts that one would have had a reasonable expectation of success in view of the results of Zolotukhin et al. and Iannacone et al. which both show that such alterations of other genes which are to be expressed in evolutionarily highly distinct organisms from those in which they evolved, substantially improve the levels of expression in the new host.

In order for the Examiner to establish a *prima facie* case obviousness, three base criteria must be met. First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. Second, there must be a reasonable expectation of success. Finally, the prior art reference (or references when combined) must teach or suggest all the claim limitations. The teaching or suggestion to make the claimed combination and the reasonable expectation of success must both be found in the prior art, and not based on applicant's disclosure. *M.P.E.P.* § 2142 (citing *In re Vaeck*, 947 F.2d 488, 20 U.S.P.Q.2d 1438 (Fed. Cir. 1991)).

None of the references alone or in combination teach or suggest a synthetic nucleic acid molecule which has a codon composition and transcription regulatory

sequence composition that is different than that of a wild type nucleic acid sequence encoding a polypeptide which has at least 85% sequence identity to the polypeptide encoded by the synthetic nucleic acid molecule, wherein the codons which differ are selected so as to result in the synthetic nucleic acid molecule having a codon composition differing at more than 25% of the codons from the wild type nucleic acid sequence and having a reduced number of transcription factor binding sequences, or a vector having a modified backbone comprising a synthetic nucleic acid molecule having a reduced number of transcriptional regulatory sequences relative to a vector comprising a parent nucleic acid sequence.

Moreover, Sherf et al. provide no motivation to further modify any gene, e.g., to further increase codon substitutions without inadvertently introducing additional transcription regulatory sequences. Moreover, the data in Iannacone et al. for BtE and BtF show that an increase in codon substitutions and a decrease in RNA destabilization sequences in a synthetic gene do not necessarily improve the transcriptional characteristics of the synthetic gene relative to the reference gene.

Accordingly, withdrawal of the § 103 rejection is respectfully requested.

Conclusion

Applicant respectfully submits that the claims are in condition for allowance and notification to that effect is earnestly requested. The Examiner is invited to contact Applicant's attorney (612-373-6959) to facilitate prosecution of the application.

If necessary, please charge any additional fees or credit overpayment to Deposit Account No. 19-0743.

Respectfully submitted,

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Date August 11, 2003 By

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CERTIFICATE UNDER 37 CFR § 1.8: The undersigned hereby certifies that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail, in an envelop addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450, on this 11th day of August 2003

Name: Dawn M. Poole Signature:

2/9/1 (Item 1 from file: 71)
DIALOG(R)File 71:ELSEVIER BIOBASE

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Directed evolution: Creating biocatalysts for the future

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An effective approach to engineering new enzymes is to direct their evolution in vitro. By mimicking key processes of Darwinian evolution in the test tube, the functions of enzymes can be explored free from the constraints of function within a living system. Efficient strategies for engineering new enzymes by multiple generations of random mutagenesis and recombination coupled with screening for improved variants have been developed. Our results with industrially important biocatalysts underscore the advantages of this 'evolutionary' approach to protein engineering. (From a talk presented at the first National Academy of Engineering 'Frontiers of Engineering' Symposium, 21 September 1995).

CLASSIFICATION CODE AND DESCRIPTION:
85.3.1 - APPLIED MICROBIOLOGY AND BIOTECHNOLOGY / ENZYME BIOTECHNOLOGY / Production and Synthesis of Enzymes

1: Proc Natl Acad Sci U S A. 1997 Jul 22;94(15):7997-8000. Related Articles, Link

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Functional and nonfunctional mutations distinguished by random recombination of homologous genes.

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We describe a convenient method for distinguishing functional from nonfunctional or deleterious mutations in homologous genes. High fidelity in vitro gene recombination ("DNA shuffling") coupled with sequence analysis of a small sampling of the shuffled library exhibiting the evolved behavior allows identification of those mutations responsible for the behavior in a background of neutral and deleterious mutations. Functional mutations are expected to occur in 100% of the sequenced screened sample; neutral mutations are found in 50% on average, and deleterious mutations do not appear at all. When used to analyze 10 mutations in a laboratory-evolved gene encoding a thermostable subtilisin E, this method rapidly identified the two responsible for the observed protease thermostability; the remaining eigh were neutral with respect to thermostability, within the precision of the screening assay. A similar approach, coupled with selection for growth and survival of the host organism, could be used to distinguish adaptive from neutral mutations.

PMID: 9223302 [PubMed - indexed for MEDLINE]

1: Proc Natl Acad Sci U S A. 1994 Oct 25;91(22):10747-51. Related Articles, Link

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DNA shuffling by random fragmentation and reassembly: in vitro recombination for molecular evolution.

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Computer simulations of the evolution of linear sequences have demonstrate the importance of recombination of blocks of sequence rather than point mutagenesis alone. Repeated cycles of point mutagenesis, recombination, an selection should allow in vitro molecular evolution of complex sequences, such as proteins. A method for the reassembly of genes from their random DNA fragments, resulting in in vitro recombination is reported. A 1-kb gene, after DNase I digestion and purification of 10- to 50-bp random fragments, was reassembled to its original size and function. Similarly, a 2.7-kb plasmid could be efficiently reassembled. Complete recombination was obtained between two markers separated by 75 bp; each marker was located on a separate gene. Oligonucleotides with 3' and 5' ends that are homologous to the gene can be added to the fragment mixture and incorporated into the reassembled gene. Thus, mixtures of synthetic oligonucleotides and PCR fragments can be mixed into a gene at defined positions based on homology. As an example, a library of chimeras of the human and murine genes for interleukin 1 beta has been prepared. Shuffling can also be used for the in vitro equivalent of some standard genetic manipulations, such as a backcross with parental DNA. The advantages of recombination over existing mutagenesis methods are likely to increase with the numbers of cycles of molecular evolution.

PMID: 7938023 [PubMed - indexed for MEDLINE]

1: Eur J Biochem. 2000 Mar;267(5):1495-501. Related Articles, Link

Assessment of amino-acid substitutions at tryptophan 16 in alpha-galactosidase.

Maranville E, Zhu A.

Laboratory of Molecular Immunology, Lindsley F. Kimball Research Institute, The New York Blood Center, New York, USA.

The tryptophan residue at position 16 of coffee bean alpha-galactosidase has previously been shown to be essential for enzyme activity. The potential role of this residue in the catalytic mechanism has been further studied by using site-directed mutagenesis to substitute every other amino acid for tryptophan at that site. Mutant enzymes were expressed in Pichia pastoris, a methylotrophic yeast strain, and their kinetic parameters were calculated. Only amino acids containing aromatic rings (phenylalanine and tyrosine) were able to support a significant amount of enzyme activity, but the kinetics and pH profiles of these mutants differed from wild-type. Substitution of arginine, lysine, methionine, or cysteine at position 16 allowed a small amount of enzyme activity with the optimal pH shifted towards more acidic. All other residues abolished enzyme activity. Our data support the hypothesis that tryptophan 16 is affecting the pKa of a carboxyl group at the active site that participates in catalysis. We also describe an assay for continuously measuring enzyme kinetics using fluorogenic 4-methylumbelliferyl substrates. This is useful in screening enzymes from colonies and determinin the enzyme kinetics when the enzyme concentration is not known.

PMID: 10691988 [PubMed - indexed for MEDLINE]

1: FEBS Lett. 2000 Jan 21;466(1):87-90. Related Articles, Link

ELSEVIER SCIENCE FULL-TEXT ARTICLE

Single amino acid substitutions affecting the specificity of the fungal ribotoxin mitogillin.

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Mitogillin and related fungal ribotoxins are small basic ribonucleolytic proteins that inhibit protein synthesis by specifically hydrolyzing a single phosphodiester bond in the universally conserved alpha-sarcin/ricin loop (SRL) of large subunit ribosomal RNAs. It was previously shown that mitogillin is a natural derivative of a T1/U2-like ribonuclease with inserted domains that are involved in target selection and specificity. Site-directed mutagenesis was used to substitute single amino acids in the previously identified functional domains Ala1-Tyr24 (B1-L1-B2 domain) and Lys106-Lys113 (L4 region). Examination of the activities of the mutants in the digestion of polyinosinic acid (a ribonuclease substrate) and specific cleavag of the SRL shows that Asn7Ala and Lys111Gln substitutions lead to altered ribonuclease activity and diminished substrate specificity consistent with the proposed functions of these domains.

PMID: 10648818 [PubMed - indexed for MEDLINE]

1: Biochem Biophys Res Commun. 1998 Oct 29;251(3):714-9.

Related Article Link

ELSEVIER SCIENCE FULL-TEXT ARTICLE

A mutation Ser213/Asn in the hexokinase 1 from Schizosaccharomyces pombe increases its affinity for glucose.

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Alignment of amino acids of the region implicated in glucose binding from a series of hexokinases showed that Schizosaccharomyces pombe hexokinase 1 had a Ser residue in a place where all other kinases had an Asn. We changed an AGT codon to AAT to place an Asn in the Ser213 position. This mutation decreased Km for glucose from 9.4 mM to 1.6 mM and the ratio Vmax (Fructose)/Vmax (Glucose) from 5 to 2.5. Also the Km for 2-deoxyglucose decreased from 2.7 mM to 0.8 mM. A mutation in the similar position of S. pombe hexokinase 2 (Asn196/Ser) increased the Km for glucose from 0.16 mM to 0.56 mM. Fermentation of glucose is not detectable in a S. pombe mutant with only hexokinase 1 activity but expression of the hxk1(S213/N) gene conferred ability to ferment the sugar. While the mutated hexokinase 1 partially mimicked S. cerevisiae hexokinase II in catabolite repression of invertase, the wild type one could not substitute for it.

PMID: 9790975 [PubMed - indexed for MEDLINE]

1: Biol Chem. 1997 Oct;378(10):1205-9. Related Articles, Link

Site-directed mutagenesis of the Cys residues in ClpA, the ATPase component of protease Ti (ClpAP) in Escherichia coli.

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The ATP-dependent casein hydrolysis by protease Ti (ClpAP) has been shown to be inhibited by sulfhydryl blocking agents, such as N-ethylmaleimide (NEM), when preincubated with ClpA but not with ClpP. To define the role of three Cys residues in ClpA, site-directed mutagenesis was performed to substitute each of them with Ser or Ala. None of the mutations showed any effect on the ATPase activity of ClpA or its ability to support the casein degradation by ClpP. However, NEM could no longer block the ability of ClpA/C47S or ClpA/C47A in supporting the ClpP-mediated proteolysis, unlike that of ClpA, ClpA/C203S, or ClpA/C243S. Furthermore, in the presence of NEM, casein could stimulate the ATPase activities of ClpA/C47S and ClpA/C47A and protect from their degradation by ClpP, but not of the other ClpA proteins. These results suggest that the inhibitory effect of NEM is due to prevention of the interaction of ClpA with casein by introduction of a bulky alkyl group to Cys47, but not linked to the catalytic function of the ATPase.

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1: J Biol Chem. 1997 May 2;272(18):11874-80.

Scanning alanine mutagenesis and de-peptidization of a Candida albicans myristoyl-CoA:protein N-myristoyltransferase octapeptide substrate reveals three elements critical for molecular recognition.

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Candida albicans produces a single myristoyl-CoA:protein N-myristoyltransferase (Nmt) that is essential for its viability. An ADP-ribosylation factor (Arf) is included among the few cellular protein substrates of this enzyme. An octapeptide (GLYASKLS-NH2) derived from a N-terminal Arf sequence was used as the starting point to identify elements critical for recognition by the acyltransferases's peptide-binding site. In vitro kinetic studies, employing purified Nmt and a panel of peptides with single Ala substitutions at each position of GLYASKLS-NH2, established that its Gly1, Ser5, and Lys6 residues play predominant roles in binding. ALYASKLS-NH2 was found to be an inhibitor competitive for peptide (Ki = 15.3 +/- 6.4 microM) and noncompetitive for myristoyl-CoA (Ki = 31.2 +/- 0.7 microM). A survey of 26 derivatives of this inhibitor, representing (i) a complete alanine scan, (ii) progressive C-terminal truncations, and (iii) manipulation of the physical-chemical properties of its residues 1, 5, and 6, confirmed the important stereochemical requirements for the N-terminal amine, the beta-hydroxyl of Ser5, and the epsilon-amino group of Lys6. Remarkably, replacement of the the N-terminal tetrapeptide of ALYASKLS-NH2 with an 11-aminoundecanoyl group produced a competitive inhibitor, 11-aminoundecanoyl-SKLS-NH2, that was 38-fold more potent (Ki = 0.40 +/- 0.03 microM) than the starting octapeptide. Removing the primary amine (undecanoyl-SKLS-NH2), or replacing it with a methyl group (dodecanoyl-SKLS-NH2), resulted in 26- and 34-fold increases in IC50, confirming the important contribution of the amine to recognition. Removal of LeuSer from the C terminus (11-aminoundecanoyl-SK-NH2) yielded a competitive dipeptide inhibitor with a Ki (11.7 +/- 0.4 microM) equivalent to that of the starting octapeptide, ALYASKLS-NH2. Substitution of Ser with homoserine

cis-4-hydroxyproline, or tyrosine reduces potency by 3-70-fold, emphasizing the requirement for proper presentation of the hydroxyl group in the dipeptid inhibitor. Substituting D- for L-Lys decreases its inhibitory activity >100-fold, while deletion of the epsilon-amino group (Nle) or masking its charge (epsilon-N-acetyl-lysine) produces 4-7-fold attenuations. L-His, but not its D isomer, can fully substitute for L-Lys, producing a competitive dipeptide inhibitor with similar potency (Ki = 11.9 +/- 1.0 microM). 11-Aminoundecanoyl-SK-NH2 and 11-aminoundecanoyl-SH-NH2 establish tha a simple alkyl backbone can maintain an appropriate distance between three elements critical for recognition by the fungal enzyme's peptide-binding site: a simple omega-terminal amino group, a beta-hydroxyl, and an epsilon-amin group or an imidazole. These compounds contain one peptide bond and two chiral centers, suggesting that it may be feasible to incorporate these element of recognition, or functionally equivalent mimics, into a fully de-peptidized Nmt inhibitor.

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1: J Virol. 1995 Dec;69(12):8173-7.

Related Articles, Link

An aspartic acid at amino acid 108 is required to rescue infectious virus after transfection of a poliovirus cDNA containing a CGDD but not SGDD amino acid motif in 3Dpol.

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The poliovirus RNA-dependent RNA polymerase (3Dpol) contains a region of homology centered around the amino acid motif YGDD (amino acids 326 to 329), which has been postulated to be involved in the catalytic activity of the enzyme. Previous studies from this laboratory have used oligonucleotide site-directed mutagenesis to substitute the tyrosine amino acid at this motif with other amino acids (S. A. Jablonski and C. D. Morrow, J. Virol. 67:373-381, 1993). The viruses recovered with 3Dpol genes with a methionine mutation also contained a second mutation at amino acid 108 resulting in a glutamic acid-to-aspartic acid change (3D-E-108 to 3D-D-108) in the poliovirus RNA polymerase. On the basis of these results, we suggested that the amino acid at position 108 might interact with the YGDD region of the poliovirus polymerase. To further investigate this possibility, we have constructed a series of constructs in which the poliovirus RNA polymerases contained a mutation at amino acid 108 (3D-E-108 to 3D-D-108) as well as a mutation in which the tyrosine amino acid (3D-Y-326) was substituted with cysteine (3D-C-326) or serine (3D-S-326). The mutant 3Dpol polymerases were expressed in Escherichia coli, and in vitro enzyme activity was analyzed. Enzymes containing the 3D-D-108 mutation with the wild-type amino acid (3D-Y-326) demonstrated in vitro enzyme activity similar to that of the wild-type enzyme containing 3D-E-108. In contrast, enzymes with the 3D-C-326 or 3D-S-326 mutation had less in vitro activity than the wild type. The inclusion of the second mutation at amino acid 3D-D-108 did not significantly affect the in vitro activity of the polymerases containing 3D-C-326 or 3D-S-326 mutation. Transfections of poliovirus cDNAs containing the substitution at amino acid 326 with or without the second mutation at amino acid 108 were performed. Consistent with previous findings, we found that transfection of poliovirus cDNAs containing the 3D-C-326 or 3D-S-326 mutation in 3Dpol did not result in the production of virus. Surprisingly,

transfection of the poliovirus cDNAs containing the 3D-D-108/C-326 double mutation, but not the 3D-D-108/S-326 mutation, resulted in the production o virus. The virus obtained from transfection of polio-virus cDNAs containing 3D-D-108/C-326 mutation replicated with kinetics similar to that of the wild type virus. RNA sequence analysis of the region of the 3Dpol containing the 3D-C-326 mutation revealed that the codon for cysteine (UGC) reverted to the codon for tyrosine (UAC). The results of these studies establish that unde the appropriate conditions, poliovirus has the capacity to revert mutations within the YGDD amino acid motif of the poliovirus 3Dpol gene and further strengthen the idea that interaction between amino acid 108 and the YGDD region of 3Dpol is required for viral replication.

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